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LLNL-TR-585512

Systems level investigation of uranium response and regulation by *Caulobacter crescentus*

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September 27, 2012

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This work performed under the auspices of the U.S. Department of Energy by Lawrence Livermore National Laboratory under Contract DE-AC52-07NA27344.

Title of Project: Systems level investigation of uranium response and regulation by *Caulobacter crescentus*

Institution: Lawrence Livermore National Laboratory

Date Received: August 2011

Principal Investigator: Dr. Yongqin Jiao

Project Results:

In the past year, we have fully started this project both in terms of laboratory setup and staffing. We have outfitted a new laboratory that hosts most of the equipment needed for the project, and we hired two postdoctoral fellows (Drs. Cho and Ma) who work on the project full time. Dr. Mimi Cho received her Ph.D. in the laboratory of Dr. JoAnne Stubbe at MIT in early 2012. Her graduate work focused on the biochemistry of polyhydroxybutyrate granule formation in *Ralstonia eutropha*. She has expertise in protein purification and enzyme characterization, which will be useful in our biochemical studies on the putative phytase. Additionally, she has experience in both fluorescence microscopy and TEM of bacterial cells, which will help us to characterize the physiology of *C. crescentus* under uranium stress. Dr. Jincai Ma has a Ph.D. in environmental sciences from Rutgers University. During his graduate studies, he employed a combination of molecular genetics, chemical kinetics, and spectroscopy to elucidate the mechanisms of selenium reduction by *Enterobacter cloacae*. After receiving his Ph.D., he studied the persistence and survival of the food pathogen, *E. coli* O157:H7, in soils of leafy greens at the US Salinity Laboratory (U.C. Riverside) for his postdoctoral training. Dr. Ma's role in the current project is to investigate the proteomics of *C. crescentus* in response to various stresses and to examine microbe-uranium interaction. Dr. Cho started at LLNL in March 2012 and Dr. Ma in June 2012. It should be noted that the PI, Dr. Jiao, took a 3-month maternity leave (Nov 2011- Jan 2012). As a result, the pace of the progress in the last year was not as fast as we had hoped. Nevertheless, we have begun several lines of experimental approaches towards the overall goal of our research: deciphering the mechanism of uranium (U) resistance in *Caulobacter crescentus*.

In the past year, our research has focused on one question: what are the genes and proteins that are involved in U (and other heavy metal) resistance in *C. crescentus* and how do these genetic elements function to confer resistance? We have started to test several of our hypotheses through a targeted approach, in which we have focused on the roles of a putative phytase and an S-layer protein. In addition, we employed three unbiased approaches towards an understanding of the fundamental processes that underpin U resistance, including Tn-seq, proteomic profiling, and Fosmid library screening. For these studies, we have integrated bacterial culturing, genetics, biochemistry, spectroscopy, electron microscopy, and next-generation sequencing. Our major accomplishments for the first year of the ECRP are listed below, categorized by specific aims.

Aim 1. Characterization of U resistance by *C. crescentus*

C. crescentus is noteworthy for its ability to survive high concentrations of U (7), but the physiology with respect to U resistance and microbe-U interaction is not well understood. We have established methods and techniques for characterizing U resistance and mineral transformation that will be useful for characterization of mutants in the future. Through growth and cell-suspension experiments, we found U resistance by *C. crescentus* is greatly dependent on medium composition, in particular, calcium and phosphate concentrations. In both rich (PYE) and minimal (M2G and M5G) media, chemical

precipitation of U-phosphate minerals dominates the system, which makes it more difficult to ascertain the biological effect on U transformation in a growth experiment. Therefore, cell suspension experiments that use a simpler and defined solution may help us distinguish biological effects more readily in future experiments.

The effect of U on the growth rate of wild type *C. crescentus* CB15N was determined in liquid PYE medium (Fig. 1). CB15N began to exhibit a growth defect starting at 400 μ M uranyl nitrate. No growth defect was observed up to 200 μ M uranyl nitrate in the PYE medium. Similar growth experiments were also performed in minimal medium M2G (high phosphate) and M5G (low phosphate). We found that U resistance (measured by change in growth rate) in both media depends largely on the concentrations of calcium (Ca) and phosphate (P). The higher the Ca and P concentration, the better the growth and resistance to U. When we lower the [P] to below 100 μ M, the concentration that is required to support normal growth, *C. crescentus* did not grow even in the presence of sub-micromolar concentrations of U. Under these conditions, it appears that growth is more dependent on P availability rather than U toxicity (12), although the bio-availability of both are interrelated through co-precipitation (see below). Because the effects of P limitation are beyond the scope of our study, no further growth experiments were done under low-P conditions.

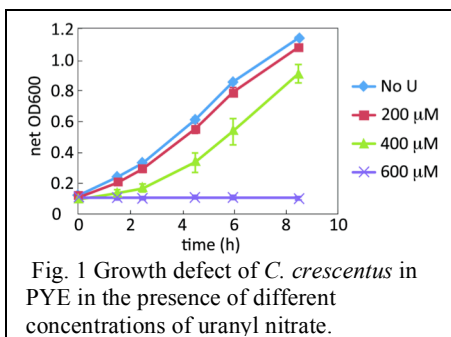


Fig. 1 Growth defect of *C. crescentus* in PYE in the presence of different concentrations of uranyl nitrate.

In both rich (PYE) and minimal (M2G) media, visible precipitation was observed immediately after U addition, indicating U mineral precipitation. Thermodynamic analysis of U speciation in M2G medium predicts that 99% of the added U is present in the solid phase (data not shown). We tested the soluble (effective) concentration of U in PYE and M2G by ICP-MS, following a growth experiment. Besides the abiotic control, we also included dead cells (killed by autoclaving) in order to distinguish U disappearance by either adsorption to cell surface or active cellular metabolism. Two concentrations of U (200 and 500 μ M) were included. The concentration of soluble U is below 45 μ M in all cultures in the presence or absence of cells. As mentioned earlier, our preliminary data suggest the effect of cells (live or dead) on U mineralogy is subtle and needs closer scrutiny. Compared to the abiotic control, the soluble U concentration was lowered (from 5-6 μ M to 1-2 μ M) with live cells, suggesting U sorption/uptake by cells. However, the soluble U concentration is higher with dead cells (10-15 μ M) compared to both live cells and the abiotic control. This observation is puzzling and needs further testing. If it holds true, the presence of dead cells may be able to inhibit chemical precipitation of U in some unknown way, which does not occur or over-ruled by other mechanisms with live cells.

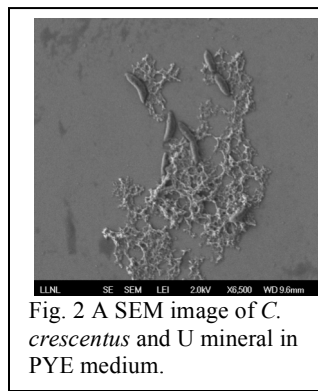


Fig. 2 A SEM image of *C. crescentus* and U mineral in PYE medium.

The composition and morphology of the U minerals formed in the presence or absence of cells were examined by SEM and XRD analyses, and will be examined by TEM in the near future. These experiments are ongoing and the results are preliminary. In PYE, U precipitates showed a poorly crystallized, net-like mineral phase (Fig. 2). In contrast, in M2G, it appeared that more crystalline, sheet-like U mineral was formed, which is characteristic of meta-autunite. XRD analysis of the minerals formed in M2G medium indicates either autunite $\text{Ca}(\text{UO}_2)_2(\text{PO}_4)_2 \cdot 10\text{-}12\text{H}_2\text{O}$ or meta-autunite $\text{Na}(\text{UO}_2)(\text{PO}_4) \cdot 3\text{H}_2\text{O}$ (data not shown). In case XRD cannot provide enough resolution about mineral identity in future experiments on the subtler, cell-induced changes to the mineralogy, we will employ XANES for U mineral analysis by working with Prof. Scott Fendorf at Stanford University.

As mentioned earlier, these basic characterizations will provide us background information towards a better understanding of U resistance physiology in wild type *C. crescentus*. Once established, we will use these methods and techniques to analyze mutant phenotypes, in order to gain an understanding of the U resistance mechanism on a molecular level. To this end, our first two targets include a phytase mutant (Δ CC1295) and a S-layer mutant (strain CB2A). The phytase story is explained below in Aim 5. S-layer proteins in *C. crescentus* and other bacteria potentially have versatile functions (9, 10) and we hypothesize that the S-layer protein in *C. crescentus* forms a protective layer on the cell surface, thus allowing cells be more resistant to U. Contrary to our hypothesis, we found that mutant lacking S-layer appeared to be more resistant to U (data not shown). Contrary to our expectations, it may be the case that the S-layer, because of its high calcium content (14) and other reasons, adsorbs U on the cell surface, rendering cells more vulnerable due to high local concentration of U, which will be tested by TEM.

Aim 2. Tn-seq screen for essential genomic elements in U resistance

Most of the experiments planned in our original proposal were based on the transcriptomic results previously conducted in Prof. Shapiro's group. However, over the course of the past year, we have found that transcription levels are often decoupled from phenotype with respect to U resistance. In other words, when mutations were made on genes that showed upregulation under U stress, we were unable to detect a U-sensitive phenotype in growth experiments with these mutants. To directly screen for genomic elements correlated with U sensitive/resistant phenotypes, we started two screens: a Tn-Seq screen and a Fosmid heterologous expression screen. The Tn-seq approach is presented here and the Fosmid heterologous expression below in Aim 3.

In our Tn-seq screening method (Fig. 3), a hyper-saturated library of transposon insertion mutants of *C. crescentus* is exposed to the selective pressure of uranium on agar plates. The transposon insertion sites of mutants that survive the U selective pressure will be sequenced via Illumina sequencing at DOE JGI, through a Quarterly Microbial Proposal awarded to the PI in May 2012. Areas of the genome with little or no insertion sites, compared to a control condition in the absence of U, would identify regions important in U resistance by *C. crescentus*.

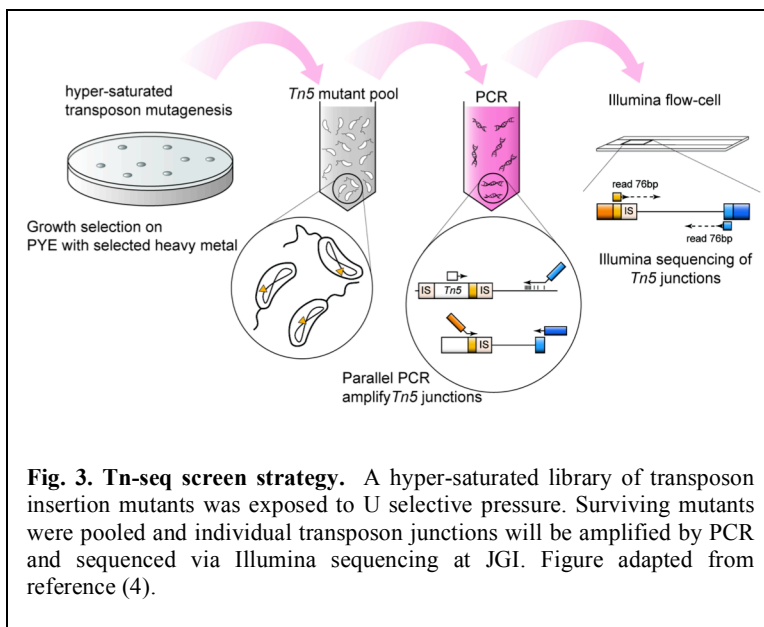
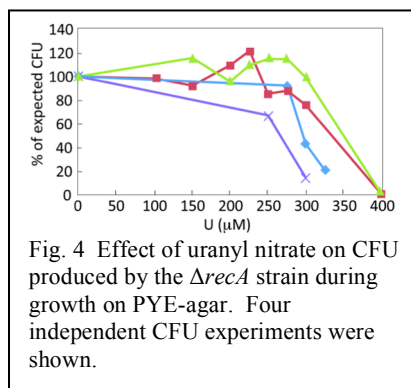


Fig. 3. Tn-seq screen strategy. A hyper-saturated library of transposon insertion mutants was exposed to U selective pressure. Surviving mutants were pooled and individual transposon junctions will be amplified by PCR and sequenced via Illumina sequencing at JGI. Figure adapted from reference (4).

We have taken several important steps in the Tn-seq screen to ensure high quality data: (1) Instead of the wild type CB15N, we used a *recA* mutant strain (Δ *recA*) in order to ensure the stability of the transposon. (2) We have included a screen condition with no selective pressure (PYE), which serves as an important reference baseline to determine the unique insertion frequency of each genomic element. Comparison of insertion frequency under no selective pressure versus U exposure is crucial for identification of U-specific elements. (3) We have included a screen condition with another heavy metal, cadmium (Cd), which serves as a positive control. A previous transposon screen of Cd-sensitive mutants in *C. crescentus* yielded several Cd-specific genes (2). Identification of these Cd-specific genes using our method would thus serve as a positive control. Additionally, we deleted one of these genes in *C.*



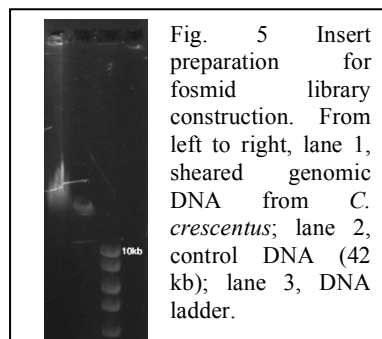
crescentus, and the resulting mutant assisted to define our Cd screen condition. Finally, the Cd condition would allow us to identify and eliminate genes that may be involved in general heavy metal stress response. (4) We tested various conditions (liquid versus solid medium with a wide range of U concentrations) to determine the optimal U screen conditions. We chose to use solid PYE-agar containing U in order to limit nutrient competition among mutants potentially encountered during liquid growth. Based on four independent CFU experiments (Fig. 4), 250 and 275 μM of uranyl nitrate were chosen as the optimal concentrations for the screen.

We have successfully generated and pooled two sets (duplicates) of one million transposon mutants under each selective condition, which comprise all of the mutants needed to complete the screen. We are currently in the process of performing two-step PCR reactions on these mutants for sequencing, according to established protocols (4). We expect to be able to send samples to JGI for Illumina sequencing in Oct. 2012. Sequence analysis will be performed in collaboration with JGI.

Aim 3. Identification of U resistance proteins by heterologous expression

Heterologous cloning has become a powerful tool to exploit the biological function of genes and proteins for the specific phenotypes (8). Through direct expression cloning, we are conducting a screen for genes from *C. crescentus* that confer U resistance in an *E. coli* host.

We have extracted high quality genomic DNA from *C. crescentus* following a protocol from JGI. The quality and quantity of the purified DNA was checked by gel electrophoresis and OD260/280. High molecular weight DNA was broken into 40 kb fragments by physical shearing (Fig. 5). A Fosmid library with average insert size of ~40 kb was constructed using the CopyControl Fosmid Library system (Epicentre, WI) from Epicenter according to manufacturer's instructions.



In order to select a host that has low U resistance as background, we tested U resistance by five *E. coli* strains, including S17-1, DH5 α , Top10, WM3064, and EPI300-T1. We found that WM3064 and EPI300-T1 are the most sensitive to U (data not shown), both of which were selected as hosts. EPI300-T1 is the host strain provided in the Fosmid Library System, in which the copy number of the Fosmid can be controlled.

To screen for U resistance phenotypes of the constructed Fosmid library, we first optimized the screen conditions using the WM3064 strain. We have tested both solid (agar) and liquid media (LB and PYE), with a wide range of U concentrations and incubation time. We found that PYE liquid medium with 250 μM uranyl nitrate after 24 h incubation gives us the largest variation in growth (OD600) among the mutants. About 600 WM3064 mutants harboring the Fosmid were screened and 20 candidates were found to show more resistance to U compared to the control. We are in the process of re-testing these candidates.

Identification of U resistance genes based on a functional screen depends on gene expression and formation of active proteins in the heterologous host. If *E. coli* is found to be a non-ideal expression host for many of the *C. crescentus* proteins, we will consider using other hosts that are phylogenetically more related to *C. crescentus*, such as *Rostonia* species or *C. crescentus* itself. For the latter, we will

consider transforming the Fosmid library back to *C. crescentus* according to an established protocol (15) and screening Fosmid-containing *C. crescentus*. In this case, we assume that the presence of one additional copy of relevant gene(s) will confer more U resistance.

Aim 4. Proteomics study of heavy metal stresses in *C. crescentus*

Previous whole-genome transcriptional profiles of *C. crescentus* under several heavy metals stresses revealed many up-regulated genes that might be responsible for resistance to those heavy metals (7). Although microarray studies can profile the overall gene expression, levels of mRNA may not be directly proportional to the expression level of the proteins they code for. Therefore, proteomic profiling of *C. crescentus* in response to heavy metal stresses has the potential to provide additional insights into resistance mechanisms, complementary to the previous global transcriptional microarray experiments.

We chose similar conditions for the proteomic profiling as the transcriptional microarray experiments (7) in order to facilitate result comparison. Cells were grown in M2G medium in the presence of different concentrations of heavy metals and salt, including U (200 and 500 μ M), cadmium (7.5 μ M), chromium (10 and 15 μ M), and salt (sodium chloride, 35 mM). The criterion for selecting the concentrations of stressors was to ensure that doubling time under stress only increased by up to 10%, which would allow for proteomic changes, but would not significantly affect growth rate (7). The actual doubling time of cells under these conditions are listed in Table 1. It is worth noting that the doubling times under U(VI) stress (200 and 500 μ M) were very similar to the unstressed control. These lower U concentrations were chosen in order to alleviate U mineral precipitation that occurs at higher U concentrations.

Cell lysis and digestion were according to established protocols (3, 13). The amounts of cells used were normalized by OD600 across samples before cell lysis. Protein concentrations (1500 μ g/ml) were determined (Bradford assay) and normalized before trypsin digestion. The protein concentrations of the trypsin-digested samples were estimated by OD280. Samples were sent to UC Davis Proteomics Core Facility for LC/MS/MS analysis in Sep 2012.

Table 1. Changes in doubling time of *C. crescentus* under various stresses.

	Control (no stress)	Salt-35	Cr(VI)-10	Cr(VI)-15	Cd(II)-7.5	U(VI)-200	U(VI)-500
Doubling time (h)	2.70	2.96	2.96	3.03	2.9	2.81	2.77
Change relative to control (%)	0.00	9.63	9.63	12.2	7.41	2.59	0.37

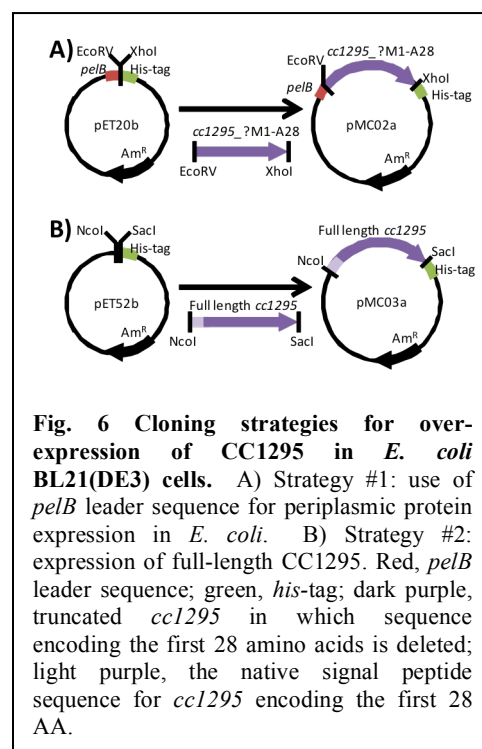
Aim 5. Examine the role of a putative phytase in U resistance

One of our objectives is to test the role of a putative phytase CC1295 in U resistance; prior transcriptional profiling studies by Prof. Shapiro and coworkers showed that CC1295 is up-regulated by >5-fold under U stress (7). We hypothesize that CC1295 serves as a nucleation point to facilitate the precipitation of a U-Ca-P complex as a result of its enzymatic activity: the breakdown of phytate to release inorganic phosphate. Ca binding to the enzyme is required for its enzyme activity (11). To test our hypothesis, we took a combination of genetic and biochemical approaches including examination of the U resistance phenotype of a *C. crescentus* mutant lacking CC1295 and enzymatic studies of purified CC1295 in relation to U precipitation in the presence of Ca and phytate.

In the genetic approach, we have tested the U resistance phenotype of a CC1295 null mutant obtained from the Shapiro lab at Stanford University. We would expect a mutant that is deficient in phytate metabolism to be more sensitive to U. In order to encourage phytase utilization conditions, we used a modified M5G medium in which inorganic phosphate was replaced with phytate as the sole

phosphate source. Contrary to our expectations, the $\Delta cc1295$ mutant exhibited no difference in growth or U resistance compared to wild type, suggesting that the mutant is not fully deficient in phytate utilization and/or that the phytate stock used had inorganic phosphate contamination, which served as a P source for cellular growth. To test the former possibility, we did a genomic search for other phytases in *C. crescentus* using the *Caulobacterbase* maintained by the University of Chicago (1) and identified another putative phytase, CC0671. If $\Delta cc1295$ is found to be able to metabolize phytate, a double mutant of $\Delta cc1295\Delta cc0671$ will be made and subsequently examined for phytate metabolism. To test the latter possibility, we determined the inorganic phosphate concentration in our two phytate stocks (Sigma) using a ferrous sulfate-molybdenum blue assay (6). We found that one phytate stock contained 30% (mol) inorganic phosphate and the other 5% (mol). We plan to purify the phytate by an HPLC method (5) and revisit the growth and U resistance experiments with purified phytate.

In the biochemical approach, we are currently in the process of protein expression and purification of CC1295. In order to over-express CC1295 in *E. coli*, we employed two cloning strategies (Fig. 6). Analysis of the CC1295 sequence by SignalP predicts that the protein has a signal peptide cleavage site, suggesting that the protein is located in the cell periphery or extracellularly. In order to promote extracellular expression of CC1295 in *E. coli*, we decided, as a first strategy to fuse a *pelB* leader sequence to the 5' end of a truncated, leader-less *cc1295*. Our second strategy is to express full-length *cc1295* with its native signal peptide. In both strategies, a gene sequence encoding a C-terminal His-tag was added for ease of protein purification. Both constructs were completed and sequenced for verification. Once the expression conditions are optimized, we will purify the protein by affinity chromatography using Ni-NTA resin. Subsequent biochemical studies will be performed to test our hypothesis that CC1295 can catalyze the precipitation of U in the presence of Ca and phytate. His-tag will be cleaved from the purified protein in case it interferes with biochemical assays.



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Products Delivered:

1. Cho M., Ma J., Bowman G, Christen B, McAdams H, Shapiro L, and Jiao Y., Identification of genomic elements involved in uranium resistance by *Caulobacter crescentus* using Tn-seq. (In preparation)
2. Cho M. and Jiao Y., The role of phosphate metabolism in uranium resistance by *Caulobacter crescentus*. (In preparation)
3. Ma J. and Jiao Y., Proteomic analysis of heavy metal stress by *Caulobacter crescentus*. (In preparation)

Program Manager: Joseph R. Graber 301-903-1239

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Lawrence Livermore National Laboratory is operated by Lawrence Livermore National Security, LLC, for the U.S. Department of Energy, National Nuclear Security Administration under Contract DE-AC52-07NA27344. Document release number: LLNL-TR-585512

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